LIMB REGENERATION AND ENDOCRINE ACTIVITY IN THE CRAYFISH[1, 2]

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With the development of the concept of neurosecretion much new information has been obtained concerning the endocrine control of growth in crustaceans. Thus, neurosecretory cells in the x organs within the eyestalk send their secretory products via their axons to swollen axon terminations which are concentrated around a blood sinus and called the sinus gland. The secretory products are stored here for later release into the blood (Bliss, 1953; Bliss, Durand and Welsh, 1954; Carlisle, 1953; Passano, 1953). One hormone of the x organ is molt-inhibiting in crabs (Passano, 1953), and evidence has been presented which indicates that it is produced in the crayfish by the type 2 neurosecretory cells in the x organ (Durand, 1956). The specific roles of other neurosecretory cells in the brain and eyestalks, some of which send their axons to the sinus glands, are unknown. Work on crustacean neurosecretion has been reviewed recently by Knowles and Carlisle (1956).

In addition to neurosecretory elements, another endocrine gland, the y organ, is important in the regulation of crustacean growth. This gland, first described by Gabe (1953), produces a hormone which has been shown to stimulate growth and molting in *Carcinides* (Echalier, 1954, 1955).

Because both of these endocrine glands function in the control of growth, it is important to know the time relationships between their periods of activity. The secretory activity of the two organs was studied, therefore, along with the regeneration of autotomized limbs since Bliss (1956) showed that certain stages in the regeneration of limbs in *Gecarcinus* reflect growth conditions within the animal in general.

Materials and Methods

Animals

Young *Orconectes limosa* (12–15 mm. carapace length) were collected from the Rahway River, Rahway, N. J. during the summer of 1956. Crayfish were then moved to Camden, N. J. and kept in half pint paraffin-lined containers, one crayfish per container. The containers were covered with translucent plastic and kept in a water bath (temperature 20–25° C.). The water in the containers was changed twice weekly during July and August and once a week during September

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and October. Freshly collected duckweed and algae, upon which the animals feed, were added to the containers each time the water was changed.

Regeneration

In one group of animals, either on the day of molt or on the day after molt, each crayfish was made to autotomize its right rear walking leg. Pinching the meropodite with fine forceps was usually a sufficient stimulus to bring about autotomy. Animals were examined for signs of regeneration of the limb about three times per week during July and August and twice a week thereafter to the middle of October. A regenerating limb grows out from the autotomy plane as a long thin flexible structure, its segments linearly arranged within a membranous sac. This membranous sac and its contents, hereafter referred to as a limb bud, was measured under 15 × magnification with a pair of dividers, and the length was taken by comparison with the half-mm. scale of a metal machininst's rule. The limb bud was measured from the tip to the plane of autotomy.

In another group, crayfish were kept in the laboratory as described above and were made to autotomize the right hind walking leg within two days of collection. A record of the regeneration of the leg was made for each crayfish as described.

In order to compare the rates of limb regeneration of different sized animals, the size of the regenerating limb is expressed as the per cent of the carapace length:

$$R = \frac{\text{length of regenerating limb}}{\text{length of carapace}} \times 100.$$

This formula is comparable to that used for studies of limb regeneration in *Gecarcinus lateralis* (Bliss, 1956).

Histology

Eyestalks and y organs were fixed in Bouin plus 1% $CaCl_2$ at various stages of limb regeneration and embedded in Tissuemat. Eyestalk serial sections (7 μ) and y organ serial sections (10 μ) were stained in most cases with the aldehyde fuchsin technique (Halmi, 1952) with modifications as suggested by Dawson (1953).

Counts of the type 2 neurosecretory cells which were located in the x organ and which contained secretory material were made according to the method given in a previous paper (Durand, 1956).

Results

Limb regeneration

Studies of the regeneration of autotomized limbs were carried out in this investigation. The rate of limb regeneration was shown earlier (Bliss, 1956) to vary at certain stages of the intermolt period, and it was hoped that limb regeneration curves might be used as an indicator of endocrine conditions in the animal in general. As will be seen, it was found that only at certain times during the intermolt period did the rate of limb regeneration reflect changes in endocrine conditions in the animal.

Typical curves of limb regeneration are shown in Figure 1. These are selected curves for individual crayfish. The animals were made to autotomize on the day

after collection, and the rate of limb regeneration was measured to the following molt. Since the animals were collected at random, it is believed that all stages of the intermolt period were represented in the population at the beginning of the experiments. Therefore, Figure 1 illustrates limb regeneration which began at successive stages of the molt cycle.

It is seen that the stages of limb regeneration are similar to those reported by Bliss (1956) for *Gecarcinus*. Thus, following autotomy there is a *lag period* before any visible signs of regeneration occur; this period probably represents the time required for healing processes and the general mobilization of local growth

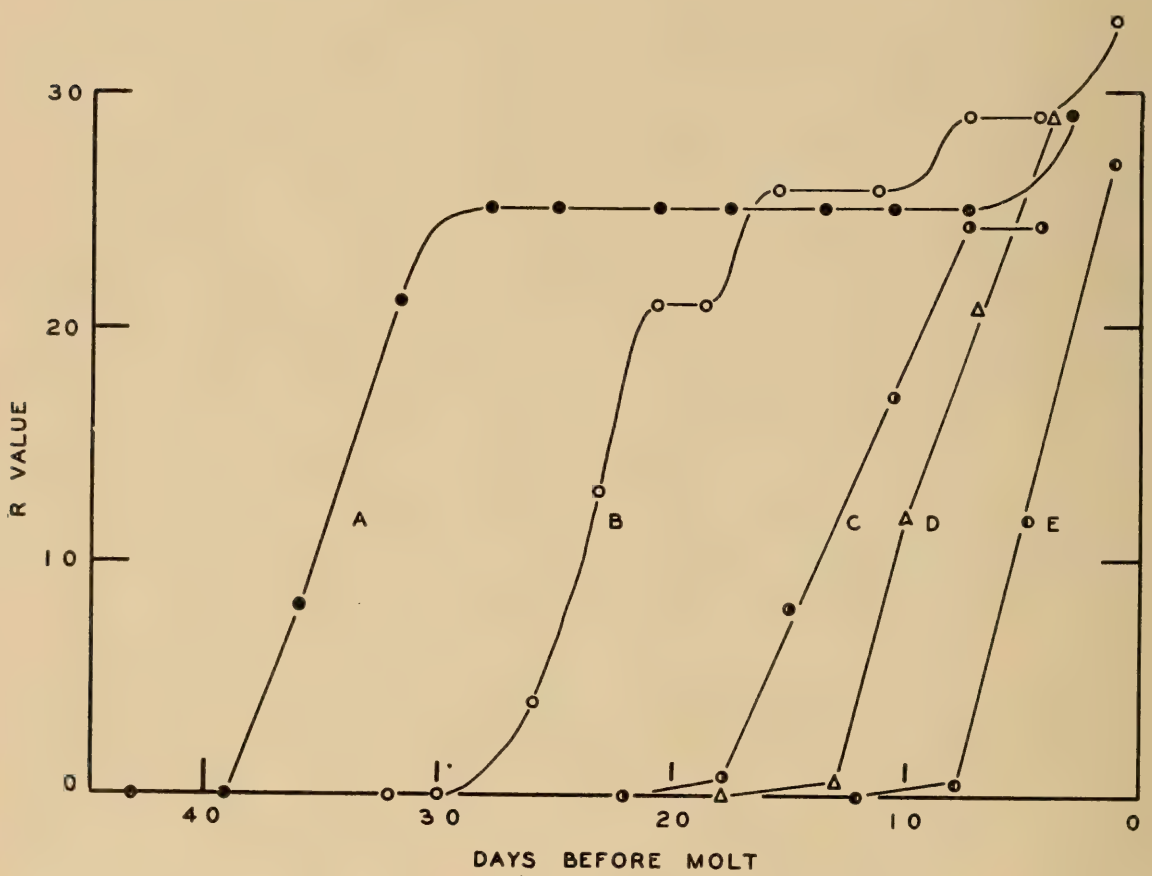


FIGURE 1. Limb regeneration curves for selected crayfish.

processes. It is interesting that the length of the lag period (mean, 6 days) is essentially constant no matter when autotomy occurs during the intermolt period.

Next, there occurs a period of *basal growth,* during which time the regeneration of the limb progresses rapidly. This stage is also essentially the same (the slopes are the same) no matter when it occurs during the intermolt period. The mean slope of the basal growth stage was 2.2, and 10–11 days were required for the completion of basal growth.

Following basal growth, linear growth of the regenerating limb ceases, that is, it enters a period of *plateau* which lasts until near the end of the intermolt period. The limb has usually reached about 22% of the carapace length at this time. According to Hodge (1956) differentiation of limb tissue continues during the period

of plateau. Therefore, only increase in length ceases while development does continue. The plateau R value is higher in *Orconectes* than it is in *Gecarcinus* (crayfish 22, crab 10). Bliss (personal communication) has suggested that this might be a reflection of the fact that the segments of the crayfish limb bud are straight whereas those of the crab are folded upon one another.

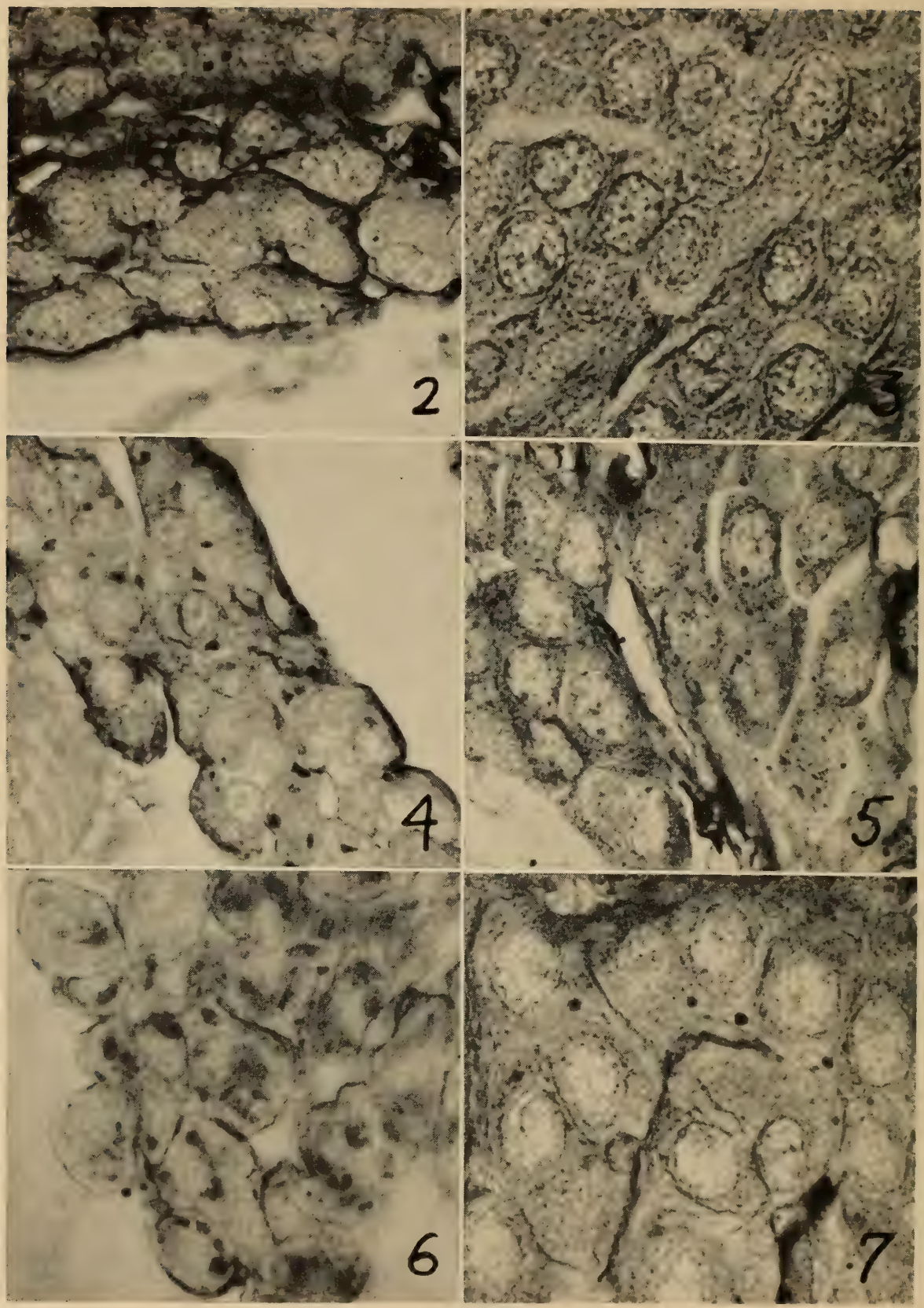
For *Gecarcinus,* Bliss (1956) reported that shortly before the animal molts growth occurs again. She termed this period *premolt growth.* Only in some instances was it possible to detect this period in the young *Orconectes* used in the present study. In a few animals a suggestion of the premolt growth stage was detected (Fig. 1, animals A and B). Since this period is often short, it is possible that it occurred between the last measurement and the molt of the animal. With a measurement interval of 3–5 days, therefore, it would be possible to detect in most cases only a slight increase, if any, in the rate of limb regeneration at the end of the period of plateau. In view of this possibility, it is interesting that some animals which were caused to autotomize passed through a period of basal growth with the normal slope. However, these animals did not show a period of plateau (Fig. 1, animals D and E). Instead, growth continued at a rapid rate, and the animals molted within a few days. It is believed that these animals had been collected very near the end of their intermolt period and that basal growth and premolt growth are continuous when autotomy occurs late in the intermolt period.

In summary, it is seen that the regeneration of autotomized limbs follows the same pattern in *Orconectes* as in *Gecarcinus.* Increase in length is restricted to the periods of basal growth and premolt growth. Since the slope of the basal growth curve remains the same at different stages in the molt cycle, it appears that this stage is not directly under endocrine control. On the other hand, premolt growth apparently is influenced by endocrine changes in the animal. Evidence for this is found later in this paper and in the study by Bliss (1956).

Y organ

A non-neurosecretory endocrine gland which functions in the control of growth in crustaceans is the y organ. Originally described by Gabe in many species of crustaceans (1953), it is located in the second maxillary segment in *Orconectes limosa.* It is always closely associated with the base of one of the large mandibular muscles and is close to, but not in contact with, the hypodermis. In serial cross-sections it lies dorso-laterally to the circumoesophageal connectives and posterior to the oesophagus. The y organ of this animal is irregular in shape; its location and rambling structure would make it difficult to extirpate.

Histologically the cells of the y organ (Figs. 2, 4, and 6) are fairly uniform in size, and very fine strands of connective tissue divide the organ into short irregular cords about 1–2 cells in thickness. Blood spaces appear in the organ but are not particularly numerous. Frequently, however, blood cells can be seen between the cords of cells. The cells of the y organ possess a very finely granular, slightly acidophilic cytoplasm with well defined cell boundaries. The nucleus is sharply outlined and usually contains a single nucleolus. Scattered in the cytoplasm of the cells the secretory material appears in the form of irregular clusters of very small aldehyde fuchsin-positive granules. When the secretory material is present in very large amounts, the cytoplasm in general is stained with aldehyde fuchsin.



FIGURES 2–7.

At these times granules or large droplets of secretory material usually are present also.

During the greater part of the intermolt period, the cells of the y organ contain moderate amounts of aldehyde fuchsin-positive material (Fig. 4). Apparently all, or almost all, of the cells possess similar amounts of secretory material at any particular time.

At molt, however, the y organ undergoes striking changes in its secretory content. Animals fixed during the first four or five days after shedding possess y organs in which it has not been possible to detect secretory material with the aldehyde fuchsin technique. The cytoplasm of these cells is apparently empty of secretory material (Fig. 2). The disappearance of the secretory material is definitely correlated with the molt of the animal although the two may not occur simultaneously. Some animals, but not all, possess empty y organs before molt. All animals possess empty y organs immediately following molt. In two cases, animals fixed while actually in the shedding process had y organs which contained more secretory material than normally (Fig. 6).

It is interesting to note that, while all animals possess secretory granules during most of the intermolt period, it is not possible to determine from y organ examination alone how near to an approaching molt the animals are. Careful study of serial sections of y organs does suggest, however, that the size of the granules in the y organ cells increases as the animals approach molt.

Neurosecretory cells

In Table I are recorded the counts of the type 2 neurosecretory cells which contain secretory material in the x organs of animals fixed at different times during the intermolt period. It is obvious that a large number of cells possess secretory material at all times except molt. Thus, from 1–2 days preceding molt to 4–5 days after molt very few type 2 cells contain secretory droplets or granules (Fig. 3). Indeed, most of the type 2 cells are devoid of all signs of secretory activity during this period. Many type 2 cells contain granules or droplets of secretory material from a few days following molt to just before the next molt (Figs. 5 and 7).

Of further interest is the observation that shortly after molt, when secretory

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FIGURE 2. Y organ from an animal during the first few days after molt. Secretory material is absent from the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

FIGURE 3. Type 2 neurosecretory cells in the x organ of an animal on the day after molt. Secretory material is absent from the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

FIGURE 4. Y organ from an animal in the basal growth stage of limb regeneration. The cells contain only a moderate amount of secretory material. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

FIGURE 5. Type 2 neurosecretory cells in the x organ of an animal in the basal growth stage of limb regeneration. The secretory material in these cells as clumps of very small granules. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

FIGURE 6. Y organ from an animal during the molt process. This animal possessed a high type 2 cell count. Note the large amount of secretory material in the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

FIGURE 7. Type 2 neurosecretory cells in the x organ of an animal during the plateau stage of limb regeneration. Note that the secretory material occurs in the form of droplets. Bouin plus calcium chloride; aldehyde fuchsin; 1140 ×.

material is again present in the type 2 cells, the material is in the form of numerous small granules scattered throughout the cytoplasm of each cell. Later in the intermolt period the secretory material is present in the form of fewer and larger droplets (Table I and Figs. 5 and 7), similar to those observed in *Orconectes*

TABLE I

Counts of type 2 neurosecretory cells which contained secretory material in the x organ

Animal number	Stage of molt cycle	Type 2 cell count	Form of material	
			Droplets	Granules
3	molting	92	+	−
39	molting	50	+	−
1	molt + hrs.	0	—	—
5	molt + hrs.	19	+	−
6	molt + hrs.	0	−	−
2	molt + 1 d.	0	−	−
58	molt + 1 d.	3	−	−
7	molt + 4 d.	25	+	−
8	molt + 4 d.	34	+	−
23	molt + 4 d.	46	+	−
24	molt + 5 d.	83	+	−
37	basal gr.	114	+	−
10	basal gr.	35	−	+
11	basal gr.	51	−	+
25	basal gr.	85	+	+
40	basal gr.	27	−	+
41	basal gr.	80	−	+
12	early plat.	70	+	−
14	early plat.	118	−	+
15	early plat.	126	−	+
17	early plat.	98	−	+
26	early plat.	70	−	+
29	early plat.	62	+	−
30	early plat.	47	+	−
31	early plat.	75	+	−
32	early plat.	74	+	−
33	early plat.	23	+	−
18	late plat.	57	+	−
19	late plat.	117	+	+
20	late plat.	66	+	−
21	late plat.	64	+	−
22	late plat.	51	+	−
42	late plat.	49	+	−
46	late plat.	31	+	−
43	premolt gr.	10	+	−
44	premolt gr.	20	+	−
45	premolt gr.	6	+	−

virilis. In an earlier study (Durand, 1956) it was suggested that the presence of secretory material in the form of small granules might represent an early stage in the production of the material. If this is so, then the absence of any signs of secretory material in the x organ type 2 neurosecretory cells in the period immediately follow-

ing molt would indicate that the synthetic activities of these cells had ceased. It is during this time that many active growth processes occur.

Discussion

So far we have seen that major changes occur in the two endocrine organs, x organ and y organ, at the time the animal molts. The secretory activity of the x organ neurosecretory cells, as indicated by type 2 cell counts, is compared to growth of regenerating limbs in Figure 8. The figure represents a generalization of the data, and an explanation of its construction is given in the next paragraph.

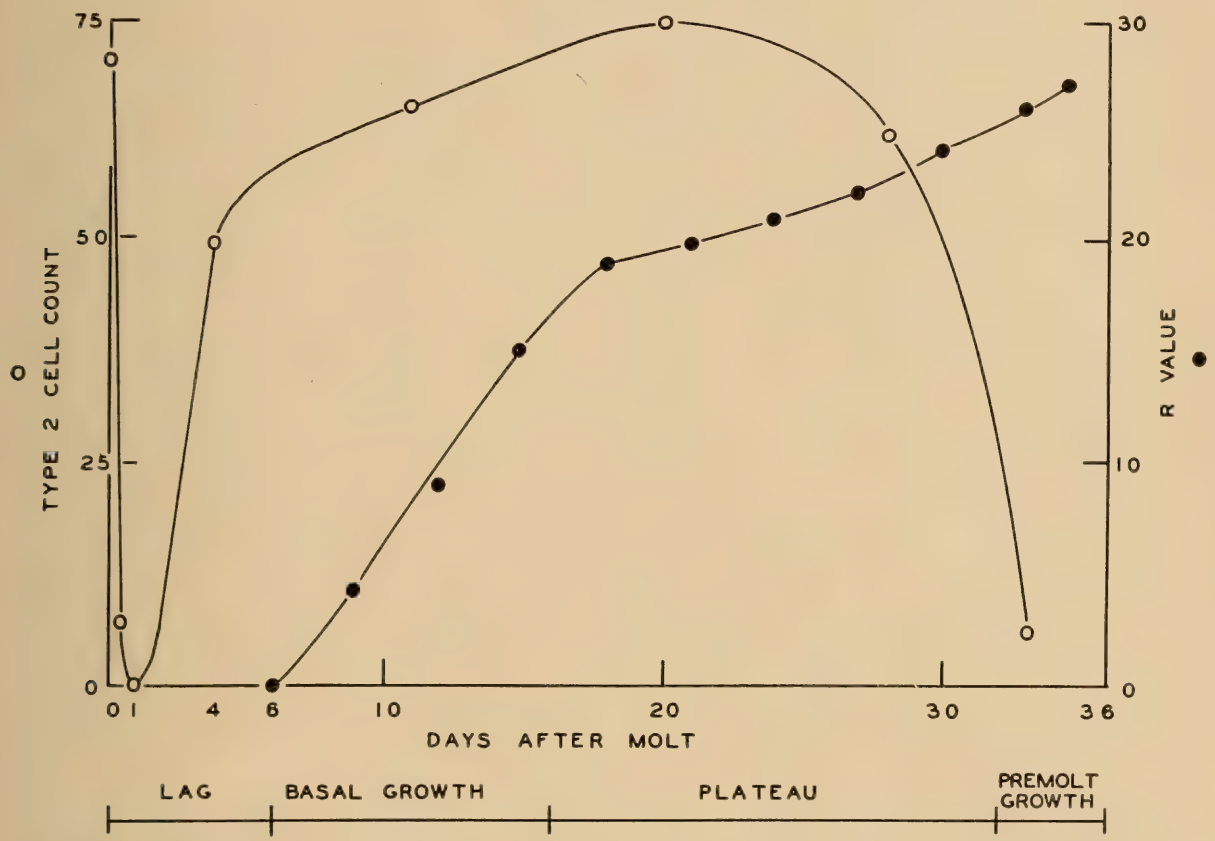

Figure 8. Type 2 neurosecretory cell counts and R values of crayfish with regenerating limbs at the indicated times during the period of regeneration.

After animals with regenerating limbs had molted, the R values for each animal were plotted against the per cent of the growth period, and smooth curves were drawn through the points. The *growth period* in this case included the time from the first appearance of a regenerating limb to the molt of the animal. From each of the curves, R values were obtained at 10% intervals of the growth period. If the mean lag period of 6 days is subtracted from the mean intermolt period of 36 days, each 10% interval in the growth period is equivalent to three days of the mean intermolt period. In Figure 8 the mean R values are plotted at each three-day interval. Also plotted are the type 2 cell counts from Table I. The mean length of time required for the completion of basal growth was 10–11 days, and the type 2 cell count for that stage was placed in the middle of that

time, on day 11 of the intermolt period. Days 16 to 32 would be the mean length of the plateau stage of limb regeneration if four days are allowed for the period of premolt growth. The mean type 2 cell counts for early and late plateau, therefore, were placed on day 20 and on day 28 of the intermolt period. These last two points represent, respectively, 25% and 75% of the plateau period. As discussed earlier, the premolt growth stage probably occurred in the last 3–5 days of the intermolt period, and so the type 2 cell count for that stage was placed on day 33, three days before molt.

Figure 8 shows that some animals possessed low type 2 cell counts during the premolt growth stage of limb regeneration and just after shedding. No high type 2 cell counts were found in animals during the first four days after shedding. In fact, the counts actually dropped to zero within 24 hours of shedding. About 4–5 days after molt the secretory material could be detected again in the perikaryon of the cells. Two notable exceptions are animals number 3 and number 39 (Table I). These animals were fixed while shedding but, nevertheless, possessed high type 2 cell counts. It should be pointed out that the y organs of these two animals, unlike those of other animals at molt, possessed unusually large amounts of secretory material. Apparently the initiation of the shedding process was independent of the y organ and x organ secretory content.

The y organ also underwent its greatest change in secretory content just prior to the molt of the animal. Thus, secretory granules were present in the y organ cells during most of the intermolt period except for a few days before and after molt. In addition the secretory granules disappeared from the y organ cells before the decrease in the type 2 cell count occurred. Thus, premolt animals were found to have high type 2 cell counts and full y organs, high type 2 cell counts and empty y organs, low type 2 cell counts and empty y organs. However, *no* animals were found to have low type 2 cell counts and full y organs.

The two endocrine organs consequently exhibited cyclical secretory behavior associated with molt. When their functions are considered, it is not surprising that they should do so. What is surprising is that both organs contained secretory material at approximately the same times during the molt cycle. On the basis of histological observations alone, it is difficult to interpret these results. For example, the presence of stainable material within a cell can mean either that the cell is producing and releasing its secretory products or that it is inactive. In the succeeding paragraphs certain physiological information concerning the two organs will be presented which suggests a possible interpretation of the observations reported here.

It is known that neurosecretory cells in the x organ produce a molt-inhibiting hormone in crabs (Passano, 1953). This hormone is passed via the neurosecretory cell axons to the sinus glands where it is stored for later release into the blood (Bliss, 1953; Bliss, Durand and Welsh, 1954; Carlisle, 1953; Passano, 1953). An earlier study by the author suggested that the type 2 neurosecretory cells in the x organ are the source of the molt-inhibiting hormone in the crayfish since they are the only neurosecretory cells that show histologically demonstrable quantitative changes in secretory material at molt. It was also suggested that the disappearance of secretory material from the type 2 cells could be accounted for by a rapid transfer of the material from the x organ to the sinus glands at molt. Another hypothesis is

that the rate of synthesis of the hormone was reduced below the rate of transfer. Since the type 2 cells are probably the source of the molt-inhibiting hormone and since the implantation of eyestalks inhibits the premolt growth stage of limb regeneration in crabs (Bliss, 1956), it is logical to assume that the presence of stainable material in the type 2 cells, during the intermolt period when molt-inhibiting hormone hypothetically is released, indicates that these cells are actively synthesizing the hormone. The absence of stainable material when molt-inhibiting hormone supposedly is witheld just before molt could result from a decreased rate of synthesis or an increased rate of transfer from the cell bodies to the sinus glands. The reappearance of the secretory material in the form of many small granules 4–5

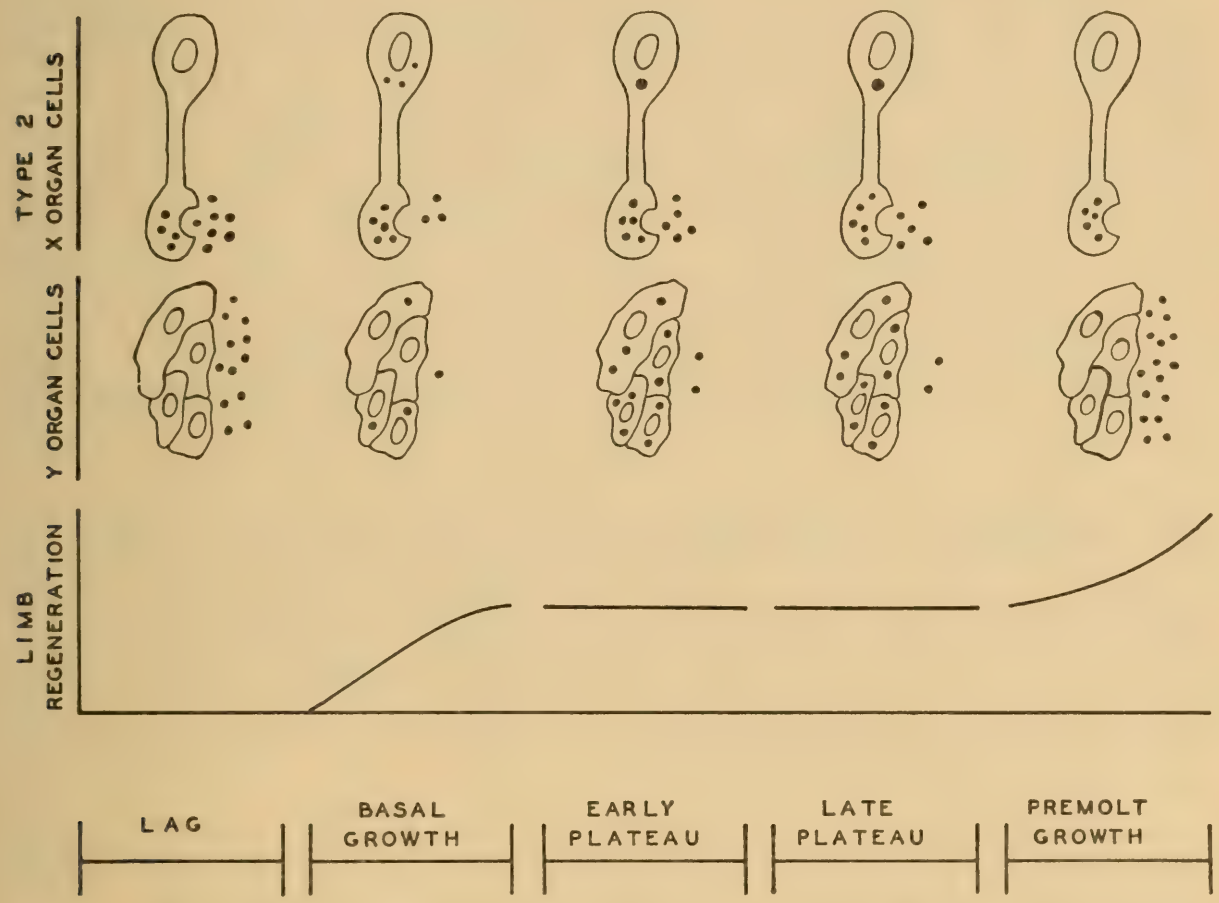

FIGURE 9. Diagrammatic summary of the secretory activity of x organ type 2 neurosecretory cells and the y organ cells in relation to limb regeneration and the molt cycle.

days after molt would result from a rate of synthesis greater than the rate of transfer from the perikaryon. Unfortunately, no information is available concerning the secretory content of the sinus glands during the stages studied in this investigation. However, Pyle (1943) has shown that the sinus glands of *Cambarus virilis* (now *Orconectes virilis*) lose most of their stainable material soon after molt.

Similar reasoning with respect to the condition in the y organ cells suggests that these cells may be actively secreting their hormone when they are histologically empty. Thus, it has been shown that the y organ is the source of a growth-promoting hormone in *Carcinides* by Echalier (1954, 1955). Travis (1955, 1957)

has shown that in *Panulirus* the resorption of the old integument occurs chiefly during the last three days before molt and the first two after molt. The production of all layers of the integument in *Panulirus* is completed by about eight days after molt. Travis reported that the intermolt period for the animals she used was 65–70 days. If these processes required a proportionate period of time in *Orconectes,* they would have occurred entirely during the period in which the y organ cells were devoid of their secretory products. For these reasons it seems logical to assume that the y organ cells are secreting their hormone when they are histologically empty at molt. However, we cannot conclude this until more is known about the physiology of the y organ and its interaction with the x organ cells.

In summary, an hypothesis can be constructed (Fig. 9) which agrees with the known facts but which requires additional experimental evidence. During the greater part of the intermolt period, secretory granules can be demonstrated in the type 2 neurosecretory cells and in the y organ cells. *Hypothetically,* molt-inhibiting hormone is released from the type 2 neurosecretory cells during this time while growth-promoting hormone is withheld by the y organ cells or released in insignificant quantities. The basal growth and plateau stages of limb regeneration can occur during this period. Just before molt and for a short time after, secretory material cannot be demonstrated in either the type 2 neurosecretory cells or the y organ cells. *Hypothetically,* the molt-inhibiting hormone of the x organ is not released at this time but the growth-promoting secretion of the y organ is released. At this time both premolt growth of regenerating limb buds and molt can occur.

Summary

1. Four periods in the regeneration of autotomized limbs can be identified in the young of *Orconectes limosa.* The first, designated *lag period,* is one in which no growth occurs and lasts about 6 days. The second, the period of *basal growth,* is characterized by rapid growth of the regenerating limb. At the end of this period the regenerating limb is about 22% of the carapace in length. During the third period, called *plateau,* no growth occurs; this period occupies the greater part of the intermolt period. In the fourth period, *premolt growth,* rapid growth again occurs about 3–5 days before molt.

2. The y organ and its secretory behavior are described. Changes in the activity of the y organ can be demonstrated histologically for only a brief period which extends from about three days before molt to 4–5 days after molt.

3. The changes in the activity of the type 2 neurosecretory cells in the x organ are described. The most marked changes occur only just before molt and persist for a period of 4–5 days after molt.

4. The secretory activity of the x and y organs are discussed in relation to the molt cycle and the regeneration of autotomized limbs. Arguments are presented in favor of the hypothesis that the y organ cells produce and release their secretory products when the type 2 neurosecretory cells in the x organ are inactive.

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